

STANFORD RESEARCH INSTITUTE

MENLO PARK, CALIFORNIA



August 21, 1964

Report No. 5
Quarterly Progress Report

SRI Project No. BU-4435
Contract No. NASR-49(11)

1 March - 1 June 1964

UNPUBLISHED PRELIMINARY DATA


FACILITY FORM 608	N 64 28935	
	(ACCESSION NUMBER)	(THRU)
	10	
	(PAGES)	(CODE)
	CR 5-8340	16
	(NASA CR OR TMX OR AD NUMBER)	(CATEGORY)

STUDIES ON THE HILL REACTION ACTIVITY OF SOLUBLE CHLOROPLAST EXTRACTS

Prepared for:

Office of Research Grants and Contracts
Code SC
National Aeronautics and Space Administration
Washington, D.C. 20546

Approved by:


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Introduction

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This study is concerned with the mechanisms and essential reactants of that part of the photosynthetic process in which oxygen is produced by photolysis of water. This is essentially the light absorbing reaction, which can be studied in vitro separately from the carbon dioxide fixing reactions. The photolysis of water and evolution of oxygen that occurs when chloroplasts or fragments of them are illuminated in the presence of a suitable electron acceptor is generally referred to as the Hill¹⁷⁻¹⁸ reaction. Attempts to reduce the Hill reaction system to a nonparticulate state have generally resulted in loss of activity. Progress toward this goal would be useful in defining the minimum components and conditions required for photolytic activity, as well as in determining the details of the mechanisms of energy transfer. Recent experiments along these lines in our laboratories led to establishment of this project to study chloroplast extracts.

✓ Au Tha

Experimental Plan

The long range plan of study includes analytical investigations to identify and characterize the components of the photoactive complex, and to determine the function of each; examination of the role of proteins, especially enzymes, in the complex; studies on methods of preparing extracts of chloroplasts, and the stability of the preparations; determination of the participation and role of various cofactors; and application of spectroscopic and other physical methods to elucidation of the characteristics of the system.

Activity During the Period

Experimental investigation concerned the development of preparative electrophoresis of chloroplast fractions in a carrier-free, high voltage unit. The electrophoretic fractions were actually tested as biochemical catalysts for the liberation of oxygen from water in the presence of light and ferricyanide. Based on specific activity, the Hill reaction activity was greater for electrophoretic effluents than for unfractionated material. Maximum activity in the effluents did not correspond to the maximum chlorophyll concentrations, indicating either inhibition by certain components or some optimal proportion of catalytic cofactors.

Future Plan

The preparative electrophoresis studies will be continued. Effluent fractions will be studied for Hill reaction activity by the highly sensitive polarographic electrode method. These studies will be complemented

by further fractionation in the ultracentrifuge. When the most active fraction is identified, it will be characterized in terms of proteins, pigments, plastoquinone, enzymes, and cofactor requirements. Methods of stabilizing and improving Hill reaction activity will be extended.

Experimental

A description of the high voltage electrophoresis separation was included in Quarterly Report No. 4. Experimental conditions were similar during the current work, except that faster buffer flow rates and consequent shorter residence times in the electrophoretic chamber were used. The following is a description of a complete experiment, beginning with the preparation of chloroplast fragments, continuing through the electrophoretic separation, and ending with the determination of Hill reaction activity by manometric methods.

Chloroplast fragments were prepared by the procedure of Spikes,²⁴ with the modifications described. The chloroplast fragments were obtained by extracting spinach leaves²⁶ with buffer identical with that used in the separating chamber of the high voltage electrophoresis unit, namely 0.025 M Tris buffer, pH 7.5. In a cold room at 4° C under dim green light, 100 g of deveined leaves was homogenized with 150 ml of Tris buffer in a Waring Blendor for about 1 min. The extract was squeezed through four layers of cheese cloth containing glass wool, and centrifuged at full speed (about 1,000 x G) in a clinical centrifuge for 1 min. The pellet of leaf tissue debris was discarded. The supernatant solution containing whole chloroplast (P₁) and chloroplast fragments (P₂) was centrifuged at full speed in a clinical centrifuge for 10 min. The pellet (P₁) was discarded.

The supernatant liquid containing chloroplast fragments (P₂) was centrifuged for 15 min. at 15,000 rpm (27,000 x G) in a Servall centrifuge. The high-speed pellets were resuspended in 20.0 ml of Tris buffer, and homogenized with a hand homogenizer. The solution was then centrifuged for 10 min at full speed in a clinical centrifuge. The pellet was discarded and a portion of the suspension of P₂ was used in the high voltage electrophoresis unit.

In contrast to curtain electrophoresis at 30 watts, a chloroplast fragment preparation can be studied easily on a preparative scale and under high power conditions at about 300 watts in the Elphor FF, as described by Hannig.¹³ The Elphor FF has no carrier; it is essentially two air-cooled glass plates about 50-cm square, separated by a 0.5-mm space through which the electrolyte passes as a film. The instrument was run at a constant current, and observed under dim green light. Forty-eight fractions divide the effluent at centimeter intervals, the fraction numbers increasing from left (anode) to right (cathode). The red pilot lights on the instrument were masked to decrease their intensity.

Concentrated Tris buffer 0.075 M, pH 7.05, was used in the electrode chambers. The electrolyte passing through the chamber was Tris 0.025 M, pH 7.05. The buffer flow rate through the separating chamber was 363 ml effluent per hour (electrolyte peristaltic pump). The dosing rate was 10.5 ml per hour (sample peristaltic pump setting). The inlet port was 34-35. With these flow rates and electrolyte concentrations, the power settings were 160 ma and 1900 volts (approximately 304 watts). The temperature control was set at 4° C. A plastic bag containing crushed ice was placed over the plastic tubing leading from the dosing pump into the chamber. The chloroplast fragment sample (P₂) was subjected to approximately 38 v/cm during about 18 min residence in the separation chamber.

The pigment was spread over a range of 7 cm in the 48 fractions, from fraction 27-34. Hill reaction activity of the fractions was determined by the usual manometric methods for oxygen evolution,¹⁴ in an Aminco Warburg apparatus or a constant volume refrigerated respirometer. All manipulations, prior to the exposure to red light during the photolysis reaction, were performed under dim green light. The temperature was maintained at 14.4 ± 0.1° C during runs. The conical Warburg vessels were illuminated with red light from below by a circular, five-tube neon bulb. The light intensity was approximately 7,000 lux. The 3-ml reaction volume in each Warburg flask consisted of 1.0 ml P₂, containing about 0.25-1.6 mg chlorophyll, 0.8 ml Tris buffer (pH 7.05), and 1 ml of Hill reaction oxidant (0.01 mg potassium ferricyanide). There was 0.2 ml of 10% KOH in the center wells. Vessels and lines were flushed with pre-purified nitrogen. Evolution of O₂ in each Warburg manometer was corrected against a thermobarometer.

The chlorophyll concentration in the Elphor FF fractions was determined according to the procedure of Arnon.¹ A 0.5-ml sample of each fraction was diluted to 25 ml in 80% acetone. The absorbance of these solutions was measured in a Beckman DU spectrophotometer equipped with a Gilford Model 220 absorbance indicator at 645, 652, and 663 mμ. The concentration of total chlorophyll, of chlorophyll a and b, and the Hill reaction activity in selected Elphor fractions are summarized in Table I. Green effluent from the Elphor FF subsequently fluoresced characteristically red when irradiated with light of wave length 365 mμ.

A second experiment involving an Elphor separation and Hill reaction determination was performed on an independent preparation of chloroplast fragments. A slightly higher electrolyte, pH 7.49, was used. The conditions in the Elphor FF separation were essentially the same, with the exception that the volts were 2100; consequently, the chloroplast fragments were subjected to 42 v/cm for about 18 min. The power was 336 watts. Data for the second experiment are shown in Table II.

The concentration of chlorophyll (a + b) in each of the effluent fractions from the Elphor was approximately 4 to 5 times as dilute as the concentration in the dosing solution (usually 1.5 mg chlorophyll/ml). Even at this dilute concentration, it was possible to use the effluent directly for the manometric measurement of Hill reaction activity without

Table I

CHLOROPHYLL CONCENTRATIONS AND HILL REACTION ACTIVITIES OF
ELECTROPHORETICALLY SEPARATED CHLOROPLAST FRAGMENTS
pH 7.05

Fraction	Chlorophyll (mg/ml)						Q Chl O ₂
	Total		Chl a	Chl b	% a	% b	
	Approx.	Precise					
Control	1.36	1.4	1.05	0.35	75.00	25.00	406
30	0.28	0.287	0.211	0.076	73.52	26.48	729
31	0.343	0.334	0.252	0.082	75.45	24.55	525
32	0.278	0.270	0.202	0.068	74.81	25.19	712

Table II

CHLOROPHYLL CONCENTRATIONS AND HILL REACTION ACTIVITIES OF
ELECTROPHORETICALLY SEPARATED CHLOROPLAST FRAGMENTS
pH 7.49

Fraction	Chlorophyll (mg/ml)						Q Chl O ₂
	Total		Chl a	Chl b	% a	% b	
	Approx.	Precise					
Control	1.60	1.39	0.910	0.480	65.5	34.5	327
27	0.33	0.291	0.183	0.108	62.9	37.1	424
28	0.44	0.389	0.245	0.144	63.0	37.0	567
29	0.41	0.368	0.233	0.135	63.3	36.7	382
30	0.294	0.263	0.153	0.110	58.2	41.8	433
31	0.267	0.238	0.148	0.090	62.2	37.8	472

a concentration step. All of the fractions showed Hill reaction activity. The middle fraction of pigmented effluent had a lower Hill reaction activity than fractions adjacent to it. This effect was noticeable in both experiments. The time required for the electrophoretic separation was approximately 90 min.

Discussion

Comparison of Hill reaction rates reported from different laboratories is difficult, if not impossible, because of the many variables. Our Hill reaction activities (Tables I and II) compare favorably with those of Becker, Gross and Shefner,³⁻⁴ who prepared crude chloroplast fragments by osmotic shock. They reported Hill reaction activity equivalent to Q_0 549, determined by the spectrophotometric method of Krogmann and Jagendorf.²² Approximately 15-30 μ g chlorophyll was present as the biocatalyst, in contrast to the milligram quantities of chlorophyll required for our manometric measurements of oxygen evolution.

Some separation of crude components occurred in our experiments. For example, electrophoretically diverted chloroplast fragments exhibited higher specific activity than did unseparated fragments. The reproducibility of lower activity of fractions from the middle region in the electrophoretic pattern is interpreted to indicate a chemically fragile fraction or one lacking components in optimal proportions. On the other hand, it should be noted that chloroplast fragments bearing chlorophyll a were not resolved from those containing chlorophyll b.

There are several useful features about the Elphor separation. Whole chloroplasts, chloroplast fragments, and even soluble components can be studied conveniently, at low temperatures, under neutral pH, and on a preparative scale. Moderately concentrated suspensions may be studied, i.e., mixtures of chloroplast fragments were applied directly into the Elphor. Some dilution was incurred by the separation, but it was not necessary to apply a concentration step, such as ultrafiltration or concentration-dialysis before the effluent could be examined for Hill reaction activity. The experiment can be performed relatively rapidly. Although the chloroplast fragments were in the electrophoretic field for only 18 min, longer residence periods are possible. At the upper limit (about 3 hr), the extreme conditions may prove useless against such fragile, biologically active materials with relatively short half-lives. In the Elphor, it is possible to use a variety of buffer systems, selecting those that provide a minimum of anionic uncoupling of the type described by Good.¹¹ Our preliminary studies with chloroplasts prepared with digitonin were reported in Quarterly Report No. 4. Buffers containing other potential stabilizing agents, such as carbowaxes, dextrans, and polyvinylpyrrolidone are compatible with the Elphor. Fredericks and Jagendorf¹⁰ recently described an extractable protein required in the Hill reaction in *Anacystis nidulans*. Carbowax 4000 or dextran and calcium ions not only preserved the Hill reaction activity but gave marked stimulation. From our experience, these factors do not appear to play a role

in the Hill reaction system of spinach chloroplast fragments because of the notable absence of tannins. However, the presence of cofactors and protective agents could be included during Elphor separations.

Until recently, the only preparative electrophoretic method involved a solid support, such as a paper curtain.⁷ Unfortunately, the chloroplast pigment-protein complex has a marked affinity for cellulose. All of our previous attempts to study the mobility of chloroplast fragments by paper strip or paper curtain electrophoresis have been unsuccessful, because the sample remains at the point of application.

The use of the Elphor FF for the separation of chloroplast fragments is not without problems. The critical sensitivity of Hill reaction catalysts to light demands that experiments be performed in dim green light. This condition was satisfied by performance of the separation during non-working hours in the evening. Plans have been made to move the instrument into a darkened laboratory. However, this creates another problem: the power requirements during a separation are greater than 2700 watts. Consequently, provision for air-conditioning is essential.

Another problem is encountered in the method of collecting effluent from the Elphor FF: from 48 small collecting wells adjacent to the chamber, effluent is periodically aspirated into larger collector tubes in an air-cooled receiver. A nitrogen, rather than oxygen, atmosphere would favor fractions with higher Hill reaction activity.

A general problem of determining Hill reaction activity is the sensitivity of the detecting system. Standard Warburg manometry is an extravagant method to study the catalytic activity of photosynthetic systems; large amounts of the bio-catalysts (chloroplast fragments containing chlorophylls, etc.) are required to demonstrate activity by oxygen evolution. Among the alternative micro methods, the platinum electrode oxygen detector of Blinks and coworkers,⁵⁻¹⁵ as employed by Fork,⁹ offers many advantages to improve sensitivity and oxygen specificity, while conserving the bio-catalyst. Consequently, our immediate objective is to change our Hill reaction analysis to this method of detection. According to Spikes,²⁴ Hill reaction activity is inversely related to the initial concentration of oxidant. Results reported by Arnon and Whatley² strongly support the suggestion that smaller quantities of reactants and catalysts should facilitate higher Hill reaction rates.

The literature contains some references to electrophoresis of pigment-protein complexes. Svensson and Brattsten²² and later Brattsten⁶ described the continuous electrophoretic separation of the algal billichromoproteins phycoerythrin and phycocyanin. Two chromoglobulins containing phosphate groups were included among 6 or 7 components electrophoretically resolved from chloroplast proteins by Sisakyan and Melik-Sarkisyan.²³ Eversole and Wolken⁸ described chloroplastin, a digitonin-extracted chloroplast fragment preparation, as electrophoretically homogeneous; however, no details were given. A description of experimental conditions and results was omitted by Heber,¹⁶ who claimed to have achieved Hill reaction activity

of small chloroplast fragments separated by curtain electrophoresis.

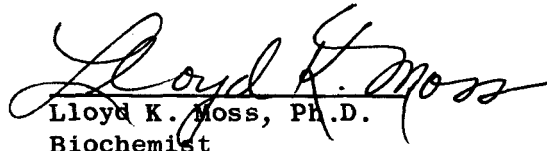
Gross, Becker, and Shefner¹² reasoned that it might be possible to isolate, from a centrifugal fraction of chloroplast fragments prepared by osmotic shock, a chemically and structurally pure fraction that might be responsible for the high Hill reaction activity of the parent mixture and that would exhibit an even higher reaction rate. The electrophoretic method of Kolin¹⁹⁻²¹ was used in attempts at further fractionation. However, the volume of the fractions withdrawn from the Kolin cell was so small that there was insufficient material for assay of both the chlorophyll content and Hill reaction activity. While the Kolin method may ultimately prove rapid, convenient, and sensitive for qualitative analysis of any biophysical separation, it is incomparable with the preparative scale afforded by the Elphor FF.

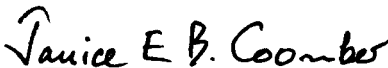
The successful electrophoretic separation of chloroplast fragments on a preparative scale adds another dimension to research concerning the Hill reaction and photosynthesis. For example, centrifugal fractions such as those described by Becker and co-workers can be studied in the Elphor FF. Within the coming months, we plan to investigate in the Elphor the centrifugal fraction equivalent to CF₂₀₋₅₀ chlorophyll/protein ratios for each fraction; the relationship of chlorophyll, plastoquinone, and protein to the Hill reaction activity, i.e., activity as a function of composition; and the storage stability, fluorescence spectra, and electron micrography of the chloroplast fragments in each fraction. Improvements in the electrophoretic separation methods will include a study of buffer systems, the optimum pH of separation, and the use of a pH gradient during electrophoresis.

Financial Status

During the fifth quarter, approximately 25 percent of the allocated funds were spent. Work will continue at about the same rate of effort for the sixth quarter.

Respectfully submitted,


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LKM(ah)

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